Use of Immunologic Markers in Obscure Hematologic Disease

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Tumor cells from two patients with very different clinical diseases were evaluated using immunologic techniques. The first patient had a persistent T-cell lymphocytosis and other symptoms suggestive of T-cell malignancy. She also had an adnexal mass. Following surgical excision, the mass was identified as a benign teratoma that contained thymic tissue. The T-cell lymphocytosis subsided following removal of the mass. The second patient had leukemia, the cells of which were morphologically identified as myelomonocytic. Surface marker studies, however, showed the presence of monoclonal surface immunoglobulins. Enzymatic digestion of the cells showed that the antibodies were cytophilic and not produced by the tumor population. These studies emphasize the value of immunologic techniques in characterizing tumor cells.

Evaluation of lymphocyte surface markers and substructures in lymphoproliferative disorders has become routine in many institutions. Typically these techniques involve such procedures as identifying T lymphocytes by E rosetting, B lymphocytes by EAC rosetting and surface immunoglobulins by fluorescence microscopy. These, along with the use of electron microscopy and immunoperoxidase evaluation of fixed tissue sections, often provide valuable insight as to the malignant cell of origin. We describe the use of these techniques in two cases in which the diagnosis of lymphoproliferative disease was a prime consideration.

Materials and Methods

Lymphocyte Preparation

Peripheral blood and bone marrow aspirates were collected in parenteral-grade heparin (Lipo-Hepin, Riker Laboratories, Inc, Northridge, Calif) and incubated with iron carbonyl (Atomergic Chemetals Co, Cable Place, NY) for 30 minutes at 37°C to facilitate removal of phagocytic cells. Solid tissues were minced into RPMI 1640 and forced through a fine-mesh wire screen. The lymphocytes from all samples were separated by centrifugation on Ficoll-Hypaque gradients.[1]

Surface Marker Studies: Sheep Erythrocyte Rosette (E Rosette) and EAC Rosettes

The procedure used is essentially that described by MacKenzie and Paglieroni.[2] A total of 200 viable lymphocytes was counted in a hemacytometer and those cells with three or more adherent erythrocytes were considered E rosette-positive. Aliquots of all preparations were cytocentrifuged, stained with Wright-Giemsa stain and evaluated microscopically.

Surface Immunoglobulin Evaluations

The technique used is essentially that of Paglieroni and MacKenzie.[3] In all, 200 viable cells were counted and the percentage of fluorescent cells determined using a Leitz Laborlux 12 fluorescent microscope.

Cells were fixed in modified Karnovsky's solution and evaluated according to standard electron microscopic procedures.[4,5]

Cytochemical and Histologic Stains

Cells were stained for specific and nonspecific esterase, periodic acid-Schiff (PAS) and myeloperoxidase using routine procedures. Tissue sections were fixed using B5 formalin and embedded in paraffin for sectioning and staining with hematoxylin and eosin. Immunoperoxidase study was done essentially as described by Sternberger.[6]

Trypsinization Studies

Aliquots of Ficoll-Hypaque-purified lymphocytes in RPMI 1640 supplemented with 2 mmol of glutamine and 15% pooled human type AB serum (decomplemented) were incubated with 2.5 mg per ml of trypsin

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for 30 minutes at 37°C.[7] The cells were gently washed with phosphate-buffered saline. Following incubation at 37°C for 24 hours, the cells were reexamined for the presence of surface immunoglobulins by the direct fluorescent technique as described above.

Patients

Report of Cases

CASE 1. The patient, a 32-year-old woman, was referred to our institution because of pruritus, urinary retention and lower extremity weakness for a week. On physical examination there was a right adnexal mass; there were no skin lesions, lymphadenopathy or hepatosplenomegaly, and there were bilateral sixth nerve palsy, lower extremity weakness and absent lower extremity reflexes. There were no pathologic reflexes. A diagnosis of encephalomyelitis was made, based on a spinal fluid protein value elevated at 165 mg per dl (negative for multiple sclerosis-associated IgG), a cell count of 268 mononuclear cells per μl and diffuse disorganization seen on the electroencephalogram. Her initial hemogram and chemistry panel showed no abnormalities. A pelvic ultrasound study confirmed the presence of a 5 by 8 cm cystic right adnexal mass. The pruritus was extremely severe. The patient was discharged two months later following significant recovery of neurologic function and rehabilitation, with a laparotomy planned for the near future. Results of the hemogram done at discharge were normal.

Six weeks later she noted "spots" on her legs consisting of multiple erythematous, nonblanching punctate lesions, 0.25 cm in diameter, on the dorsum of both feet. A skin biopsy specimen showed mild perivascular lymphocytic infiltration. The intense pruritus persisted and lymphocytosis was first noted in the blood. There were no other skin lesions, lymphadenopathy or splenomegaly. Bone marrow examination showed numerous aggregates of morphologically mature lymphocytes. The differential diagnosis of a lymphoproliferative disorder versus a hyperimmune reaction to the viral agent of the encephalitis was entertained and specimens of peripheral blood and bone marrow were submitted for specific lymphocyte studies. No intranuclear or intracytoplasmic viral inclusions were identified. Viral cultures and serologic testing failed to identify an etiologic agent. A laparotomy was done.

CASE 2. The patient, a 40-year-old woman, was referred to the University of California, Davis, Medical Center with a diagnosis of probable B-cell lymphoma with IgGκ marking cells. Three months before admission she had noted increased fatigue. A labial abscess developed two months later. Two weeks before admission she had swollen gums, sore throat and night sweats. At that time a hemogram showed severe anemia, with a platelet count of 30,000 per μl; the leukocyte count was 30,000 per μl with monoblastoid and mononuclear-appearing cells. Specimens of bone marrow aspiration and lymph node biopsy showed infiltration of mononuclear cells, with IgGκ immunoglobulins on the surface of cells from the node.

On transfer to hospital she was febrile, in a toxic state and had gingival hyperplasia and multiple cervical lymph nodes up to 3 cm in diameter. Hepatosplenomegaly was not noted. Her hemoglobin was 8 grams per dl, platelet count 30,000 per μl and her leukocyte count was 80,000 per μl. Morphologically, most cells appeared to be monocytes. Repeat aspiration of bone marrow showed replacement by malignant mononuclear cells. The differential diagnoses were acute myelomonocytic leukemia versus lymphoma. Specimens of peripheral blood and bone marrow were submitted for special stains and immunologic studies.

Results, Case 1

Lymphocyte Surface Marker Studies

Table 1 shows the results of sequential surface marker evaluations done on specimens of peripheral blood, bone marrow and ovarian mass. All tissues examined showed a striking increase in cells that formed rosettes at 4°C ($E^{4°}$) and, with the exception of the peripheral blood from week 13, an increase in the number of cells forming E rosettes at 37°C ($E^{37°}$). Further, the ovarian mass had no mononuclear populations that exhibited either surface immunoglobulins or a receptor for the third complement component. The mass did, however, have 46% of the cells forming E rosettes at 4°C and 78% formed E rosettes at 37°C. Bone marrow lymphocytes obtained during week 2 showed a pronounced increase of $E^{4°}$-positive cells (42%) and a modest elevation in $E^{37°}$ (11%). Figure 1 shows the absolute lymphocytosis seen three weeks before the surgical procedure (4,260 lymphocytes per μl), one week before (3,465 lymphocytes per μl), ten days after (3,132 lymphocytes per μl) and eight weeks after operation (3,150 lymphocytes per μl). The absolute number of E-rosetting cells at 4°C seen in Figure 1 are as follows: 2,811

TABLE 1.—*Lymphocyte Surface Markers in Case 1*

Time	Specimen	Rosettes %			Surface Immunoglobulins %						
		$E^{4°}$	$E^{37°}$	EAC	Polyvalent	G	A	M	D	κ	λ
Week 1	PB	66	9	1	8	3	1	3	1	5	4
2	PB	75	22	0	8	4	2	5	3	4	3
5.5	PB	83	6	11	7	2	1	2	1	3	3
13	PB	73	4	12	10	2	1	2	1	3	3
Month 2	BM	42	11	4	10	10	2	3	1	9	3
4	OM	46	78	0	1	0	0	0	0	0	0
Control	PB	55	2	21	20	4	3	8	8	12	8
Control	BM	11	2	30	35	10	1	15	11	15	7

PB = peripheral blood, BM = bone marrow, OM = ovarian mass

lymphocytes per μl three weeks before, 2,598 lymphocytes per μl one week before, 2,599 lymphocytes per μl ten days after and 2,299 lymphocytes per μl eight weeks after operation. The following absolute number of lymphocytes form E rosettes at 37°C: 383 lymphocytes per μl three weeks before, 763 lymphocytes per μl one week before, 187 lymphocytes per μl ten days after and 31 lymphocytes per μl eight weeks after surgical procedure.

Bone Marrow Cytology

Examination of bone marrow aspirates showed an active marrow, with 65% of the marrow containing appropriately maturing blood cells. Megakaryocytes were mature and present in normal numbers. The granulocytic and erythrocytic series were active and showed normal maturation. The granulocyte-erythrocyte ratio was normal.

Some areas of the marrow showed a lymphocytic infiltration consisting of some young, but mostly mature, cells. No lymphoblasts were seen. Stainable iron was present in normal amounts. The final impression was that of marrow showing a lymphocytic infiltrate.

Ovarian Mass Pathology

Gross examination of the ovarian tumor specimen showed a yellow-white mass having a rubbery consistency; a representative sample was fixed in B5 formalin. Microscopic examination of multiple sections stained with hematoxylin and eosin showed the neoplasm to be composed of benign elements of lymphoid, cartilaginous and mucosal tissue. Some of the mucosa was thin and attenuated, showing areas of mucin production. The lymphoid tissue was lobular and had a thymic cortical and medullary delineation (Figure 2-A). Well-formed Hassall's corpuscles were not identified. A small focus of pale epithelial cells with a parathyroid morphology was noted in the thymic tissue (Figure 2-B). There was a moderate cortical hyperplasia of the thymus and pale macrophages scattered throughout the cortex (Figure

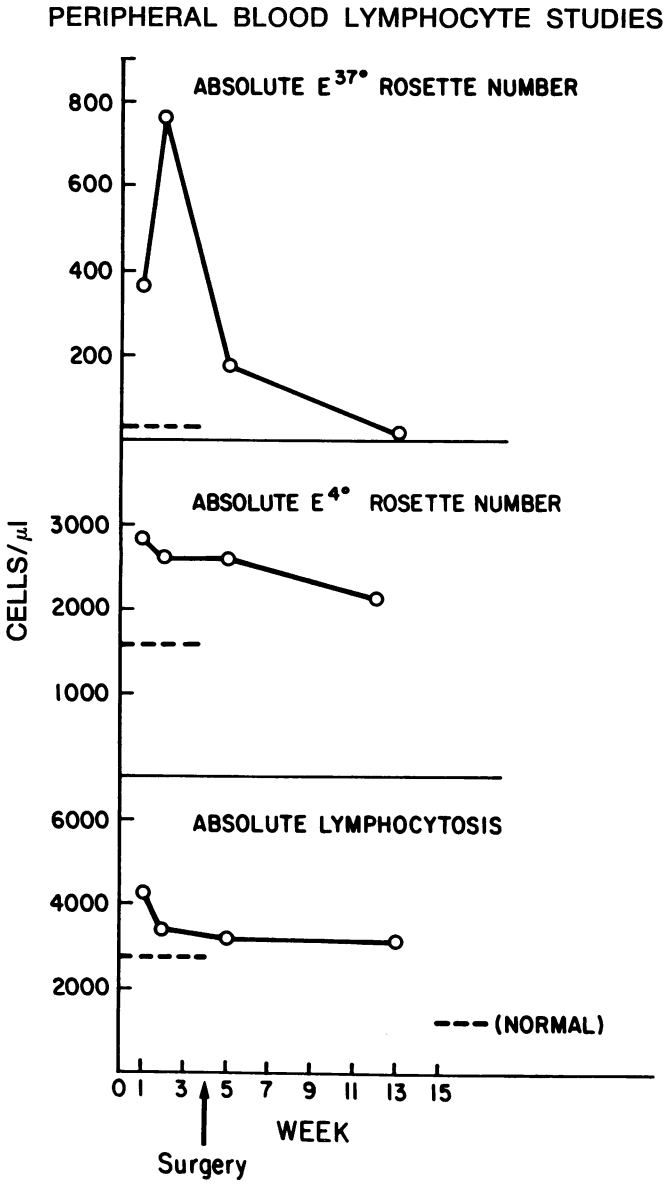

Figure 1.—Absolute peripheral blood lymphocyte values on cells from case 1. Cells had been evaluated for rosette-forming capability with sheep erythrocytes at 37°C ($E^{37°}$) and 4°C ($E^{4°}$).

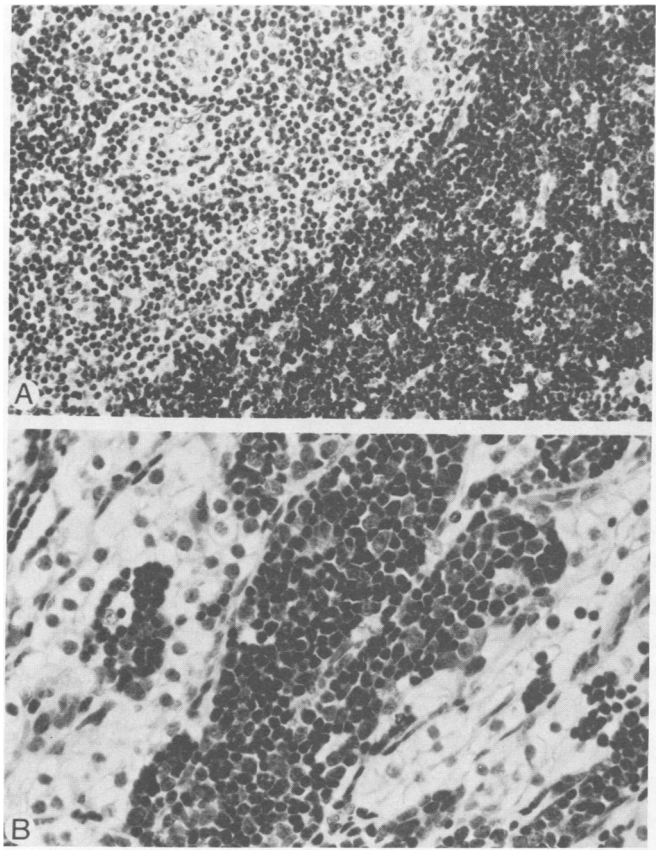

Figure 2.—**A,** Low-power magnification (reduced from ×40): Hematoxylin-eosin-stained section of benign ovarian teratoma from case 1. Compactly arranged cells are seen in the thymic cortex to the right and on the left loosely arranged cells are found in the thymic medulla. **B,** High-power magnification (reduced from ×100). Cords of pale epithelial cells with parathyroid morphology are found separating thymic lymphocytes.

2-B). In occasional medullary fibrous cords a few myeloid cells were present. No malignant elements were identified. The pathologic diagnosis was right ovarian teratoma, benign.

Results, Case 2

Morphology and Cytochemistry

Studies of peripheral blood (Figure 3-A) and bone marrow (Figure 3-B) showed infiltration by malignant cells thought by various observers to be monocytic. Results of PAS, myeloperoxidase and specific esterase stains were negative. In all, 65% of the cells stained with nonspecific esterase.

Electron Microscopy

Low-magnification electron micrographs (Figure 4-A) show the malignant cells. Nuclei are irregular in profile, containing small amounts of diffusely clumped chromatin and prominent nucleoli. The cytoplasm is abundant and contains mitochondria and large numbers of electron-dense granules. At higher magnification (Figure 4-B) the nuclear structure and nucleoli are more evident. One cell contains a prominent Golgi region. The cytoplasmic granules are varied and morphologically show a nonlymphoid structure. Some granules are very similar to those seen in monocytes.

Immunoperoxidase

Immunoperoxidase studies done on bone marrow showed only scattered cells containing IgGκ. Most of the cells, however, stained strongly for lysozyme.

Surface Marker and Trypsinization Studies

Peripheral blood mononuclear cells were analyzed for surface markers in our laboratory and that of another facility (Tables 2 and 3). Comparable data were obtained on peripheral blood values from both institutions. Results from both laboratories showed that the number of $E^{4°}$-rosetting cells in the peripheral blood was greatly reduced (30% and 21%). In addition, both sets of data show a large population of cells that stained for IgG (37% and 58%) and κ (42% and 49%). Evaluation of Ficoll-Hypaque-separated bone marrow cells showed 53% IgG and 40% κ-bearing cells. Following trypsinization and incubation of the gradient-purified mononuclear cells, the presence of surface immunoglobulins was as follows: cells bearing polyvalent immunoglobulins were reduced from 54% to 2%, IgG from 58% to 2% and κ from 49% to 2%.

Discussion

These two patients had very different clinical diseases. In each it was necessary to consult the clinical immunology laboratory for assistance in diagnosis.

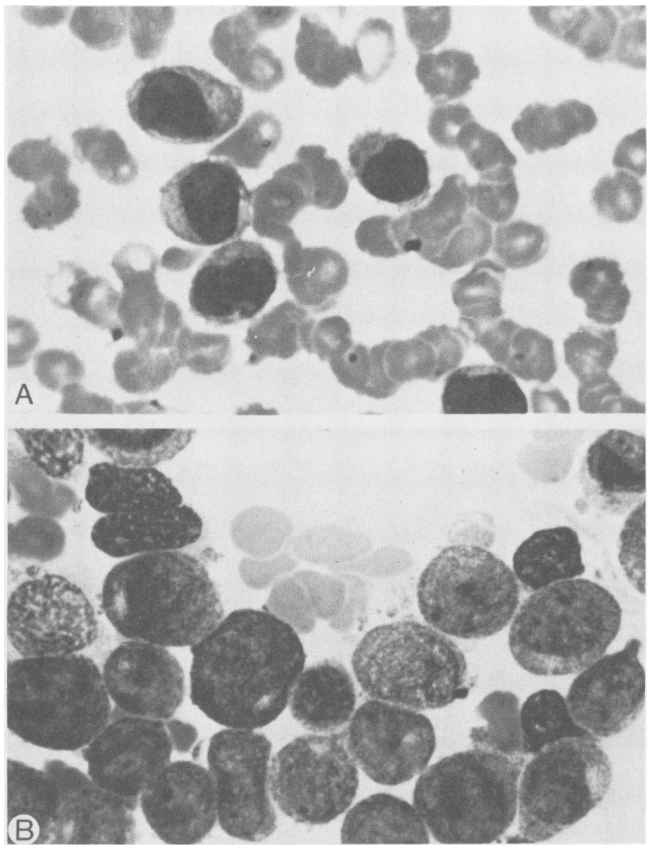

Figure 3.—A, Wright-Giemsa-stained peripheral cells from case 2. (Reduced from magnification ×250). **B,** Wright-Giemsa-stained bone marrow aspirate from case 2. (Reduced from magnification ×250.)

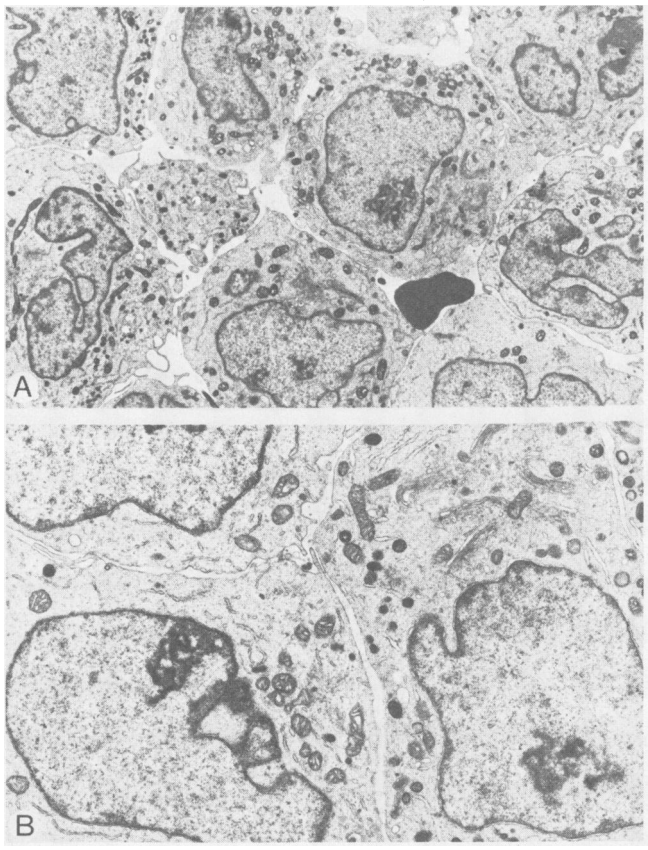

Figure 4.—A, Electron micrography of peripheral cells from case 2. (Reduced from magnification ×4,700.) **B,** Electron micrography of peripheral cells from case 2. (Reduced from magnification ×10,000.)

TABLE 2.—*Lymphocyte Surface Markers, Case 2*

Specimen	Rosettes %			Surface Immunoglobulins %						
	$E^{4°}$	$E^{37°}$	EAC	Polyvalent	G	A	M	D	κ	λ
Patient										
BM	3	<1	7	55	53	1	0	0	40	12
PB	30	<1	31	44	37	1	2	3	42	14
Control										
BM	11	<1	30	35	10	1	15	11	15	7
PB	55	<1	21	20	4	3	8	8	12	8

BM = bone marrow, PB = peripheral blood

TABLE 3.—*Effects of Trypsin on Peripheral Blood Lymphocyte Surface Markers in Case 2*

Treatment	Percent Rosettes			Percent Surface Immunoglobulins						
	$E^{4°}$	$E^{37°}$	EAC	Polyvalent	G	A	M	D	κ	λ
None	21	2	41	54	58	0	2	1	49	2
Trypsin*	..	..	..	2	2	..	..	..	2	..

* Cells treated with trypsin, incubated, then reexamined.

The first patient had a severe, life-threatening viral disease from which she was recovering when significant lymphocytosis developed. The lymphoid aggregates in the marrow, the skin lesions and the intense pruritus raised the question of malignant T-cell disease. This impression was strengthened by the finding of increased levels of $E^{4°}$ and $E^{37°}$ markers, confirming T-cell proliferation. It was expected that the right adnexal mass would be a T-cell lymphoma. Pathologically, it was a benign teratoma that included a sizable amount of normal-appearing thymic tissue, which was confirmed by histologic and immunologic studies. The presence of thymic tissue in a teratoma, though rare, has been observed by other investigators.[8] The peripheral blood lymphocytosis persisted for several weeks postoperatively. It is known that T cells are long lived, and the persistence of the $E^{4°}$ markers suggests that the lymphocytosis was of T cells. The pronounced drop in $E^{37°}$ postoperatively could be attributed to loss of functional thymic mass in the teratoma. It is known that thymic cells form E rosettes at both 37°C and 4°C, whereas mature unstimulated peripheral T cells rarely form E rosettes at 37°C.

It is not known whether the thymic cells from the teratoma were circulating in the peripheral blood or whether the thymic mass induced systemic T-cell proliferation, or both. At present we have elected to observe the patient and not institute therapy for possible T-cell hyperplasia. We speculate that we have seen peripheral blood T-cell proliferation induced by thymic tissue in a benign teratoma. If so, it is of great interest to note that skin lesions and pruritus appear to have been induced by a benign proliferation. Both have disappeared since the operation.

The second patient had a more acute problem. She was referred with a diagnosis of malignant lymphoma with monoclonal antibodies on the cells. Clinically and morphologically she appeared to have an acute myelomonocytic leukemia. The cytochemistry studies showed increased staining with nonspecific esterase and absence of staining with PAS and specific esterase. This suggested monocytic disease, a suspicion strengthened by the finding of serum lysozyme levels 20 times normal. Yet, it was puzzling that the cells marked so strongly with immunoglobulin. The trypsinization studies were invaluable in that they showed that the antibody was cytophilic and not produced by the malignant cells (Table 3). This patient received appropriate therapy for acute myelomonocytic leukemia. Cytophilic antibodies are not commonly identified in this type of leukemia, though polyclonal immunoglobulins have been reported on normal and malignant granulocytes.[9] Cell surface immunoglobulins are not usually investigated in the presence of acute myelomonocytic leukemia.

The monoclonality of the antibody is intriguing. Theoretically, it could represent an antibody directed against a specific leukemic antigen, adhesion of nonspecific antibody to malignant cells bearing very specific immunoglobulin receptors or the presence of a subset of monocytes whose Fc receptor was specific for one immunoglobulin class. Further studies are needed to clarify the significance of this finding.

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